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PLANT TRANSCRIPTION FACTORS AND ENHANCED GENE EXPRESSION

This application claims priority to U.S. Provisional Application Serial Nos. 60/201,182 and 60/266,920, both of which are expressly incorporated by reference herein.

Field of the Invention

The invention relates to transgenic plants which demonstrate enhanced expression of one or more plant transcription factors, expressed under the control of a seed specific promoter. The invention further relates to methods for producing transgenic plants which exhibit enhanced expression of one or more plant transcription factors and the use of the plants to enhance the expression level of a heterologous protein in the seeds of such transgenic plants.

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Background of the Invention

Attempts to control gene activity and/or increase the production of recombinant proteins in plants have been made using high level constitutive promoters, inducible promoters, tissue-specific promoters and developmental stage-specific promoters.

Systems for regulatable expression of genes have been reported in the literature and are generally based on modifying the activity of transcriptional regulatory proteins or by the use exogenous inducers (*i.e.*, compounds) that specifically interact with a particular transcriptional regulatory protein. In either case, the result is to modify promoter activity by affecting the binding of transcriptional regulatory proteins to their DNA binding site and thereby controlling promoter activity for a given gene.

In addition, significant research efforts have been directed to constructing improved expression vectors, modifications of promoters and modifications of 5' and 3' untranslated sequences, with the goal of increasing the expression level of heterologous protein coding sequences. The regulated expression of transgenes in plants such that expression takes place in a manner that does not result in harm to the plant is the focus of extensive research.

Cereal seed storage proteins are a primary source of proteins in the diet of humans and agricultural animals worldwide. The bulk of cereal seed storage proteins are produced solely in the endosperm, a highly specialized tissue devoted to starch and protein biosynthesis and storage. Accordingly, cereal seed storage proteins are an attractive target for the regulated expression of transgenes and a need exists for effective strategies to enhance and regulate gene expression in seed tissues of cereal plants.

Summary of the Invention

The invention provides transgenic seed crops with improved capacity to produce peptides or polypeptides by the regulated expression of transgenes in the seeds of such crops.

More specifically, the invention provides transgenic monocot plants which comprise the heterologous nucleic acid coding sequence for one or more plant transcription factors operably linked to a seed specific promoter, wherein expression of the transcription factor(s) in a plant cell is effective to activate transcription of a gene operably linked to a seed specific promoter with which the one or more transcription factors interact.

The promoter with which the transcription factor interacts may be derived from the same or a different species from plant in which it is expressed.

The seed specific promoter may be a native or heterologous seed-specific promoter. In some cases, the transcription factor also activates its own promoter.

In one aspect, the transgenic monocot plant comprises the coding sequence for more than one plant transcription factor, each of which is operably linked to a seed specific promoter. The operably linked seed specific promoters may be the same or different.

In a related aspect, the same transcription factor is expressed under the control of two or more different seed-specific promoters, which may result in expression in different seed tissues.

In yet another related aspect, the invention provides transgenic plants that contain the heterologous nucleic acid coding sequence for more than one transcription factor, wherein expression of the transcription factors results in an additive enhancement of expression of a gene operably linked to a seed-specific promoter with which the transcription factors interact.

Preferred transcription factors include opaque 2 (O2), prolamin box factor (PBF), and the rice endosperm bZIP protein (Reb).

Preferred seed-specific promoters include the Gt1, *Glb, Bx7, RP6* and *PG5a* promoters, having the sequence presented as SEQ ID NO:26, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:27 and SEQ ID NO:28, respectively.

Preferred plants include rice, corn, barley and wheat.

The invention further provides methods for producing transgenic monocot plants and seeds comprising the heterologous nucleic acid coding sequence for one or more plant transcription factor(s) operably linked to a seed specific promoter. Such transgenic plants may also include the coding sequence for a selected heterologous protein operably linked to a seed-specific promoter that is responsive to the plant transcription factor(s), such that the transgenic plant exhibits enhanced expression of the selected heterologous protein.

The invention also includes a method for producing a transgenic plant that expresses a selected heterologous protein by crossing a transgenic plant that expresses the heterologous nucleic acid coding sequence for one or more plant transcription factors operably linked to a seed

specific promoter with a transgenic plant containing a heterologous protein coding sequence under the control of a seed-specific promoter that is responsive to the one or more plant transcription factors.

In yet another aspect, the invention provides a method of making a seed-specific promoter responsive to a transcription factor to which it does not respond in its native state, a modified seed-specific promoter prepared by the method and a transgenic plant comprising such a modified seed-specific promoter.

Brief Description of the Figures

Figures 1A-F illustrate cereal seed morphology, wherein: Fig 1A provides a schematic depiction of cereal seed morphology; Fig 1B a schematic depiction of the life cycle of the cereal seed; Fig 1C is an image of GUS (β-glucuronidase) expression in the endosperm of the seed (production in starch); Fig 1D is an image of GUS expression in the embryo of the seed (production in germ); Fig 1E is an image of GUS expression in the alleurone of the seed (production in bran); and Fig 1F is an image of GUS expression during germination (production in malt).

Figures 2A-I are a double-stranded depiction of the DNA sequence of the rice (*Oryza sativa*) bZIP protein, designated ("Reb"). The gene sequence of 6.227 kb consists of 5 introns and 6 exons flanked by 1.2kb of the 5' promoter and 1.2 kb of the 3' region.

Figure 3 presents a restriction map of the rice Reb gene isolated from BAC clone 42B9.

Figure 4 is a single-stranded depiction of a portion of the DNA sequence of the Glb promoter with putative Reb binding sites indicated.

Figures 5A-C present a schematic diagram of 3 plasmids which contain the Reb coding sequence under the control of (A) the globulin promoter (Glb), (B) the actin promoter (Act) and (C) the native Reb promoter.

Figure 6A presents a schematic diagram of the plasmid constructs used for transient assays illustrating the transactivation function of the Reb gene towards the Glb promoter where (1) is a GUS reporter construct with GUS expressed under the control of the Glb promoter (Glb-GUS-Nos); (2) is a null promoter construct (Δpromoter-Reb-Term) where the cells were co-bombarded with the Glb-GUS reporter gene construct; (3) is an Reb expression construct with Reb expressed under the control of the Glb promoter (Glb-Reb-Term), where the cells were co-bombarded with the Glb-GUS-Nos reporter gene construct; (4) is an Reb expression construct with Reb expressed under the control of the Actin promoter (Act-Reb-Term) where the cells were co-bombarded with the Glb-GUS-Nos reporter gene construct; and (5) is an Reb expression construct with Reb expressed under the control of the native Reb promoter (Native-Reb-Term), where the cells were co-bombarded with the Glb-GUS-Nos reporter gene construct.

Figure 6B illustrates the relative GUS activity as measured in transient expression assays using the effector/reporter combinations shown in Figure 6A.

Figure 7A presents a schematic diagram of the plasmid constructs used for loss-of-function analysis of Reb transactivation measured by a transient assay using: (1) the GUS reporter construct, Glb-GUS-Nos; (2) the Native-Reb-Term construct co-bombarded with the Glb-GUS-Nos reporter construct; (3) the GlbΔUAS-GUS-Nos construct; and (4) the Native-Reb-Term co-bombarded with the Glb reporter construct in which the Reb UAS motifs of the Glb promoter [GCCAGT(A/C)AG] were deleted.

Figure 7B illustrates the relative GUS activity as measured in transient expression assays using the effector/reporter combinations shown in Figure 7A.

Figure 8A presents a schematic diagram of the plasmid constructs used for gain-of-function analysis Reb transactivation by transient assay using: (1) a GUS reporter construct with GUS expressed under the control of the Gt1 promoter (Gt1-GUS-Nos); (2) the Gt1-GUS-Nos construct where the cells were co-bombarded with an expression construct which has Reb expressed under the control of the native Reb promoter (native-Reb-Term); (3) a GUS reporter construct with GUS expressed under the control of the Gt1 promoter modified to contain the Reb response sequence, UAS (Gt1-UAS-GUS-Nos); and (4) the modified Gt1-UAS-GUS-Nos reporter construct where the cells were co-bombarded with native-Reb-Term.

Figure 8B illustrates the relative GUS activity as measured in transient expression assays using the reporter constructs shown in Figure 9A.

Figure 9 depicts the DNA sequence of the rice (*Oryza sativa*) globulin promoter, ("Glb") with putative binding sites for the O2 transcription factor and the prolamin box indicated in the figure.

Figure 10 depicts the DNA sequence of the wheat Bx7 promoter with putative binding sites for the O2 transcription factor and the prolamin box indicated in the figure.

Figures 11A-C provide a schematic depiction of the map of 3 plasmids which contain various transcription factor coding sequences under the control of the rice endosperm-specific glutelin promoter (Gt-1), where (A) the plasmid includes the barley prolamin box binding factor protein (BPBF), (B) the plasmid includes the maize prolamin box binding factor protein (PBF) and (C) the plasmid includes the maize opaque2 binding protein (O2).

Figure 12A illustrates reporter (Glb/GUS/NOS, RP6/GUS/NOS, PG5a/GUS/NOS and Bx7/GUS/NOS) and effector (*Ubi:O2* and *Ubi*:PBF) plasmids used in transient expression assays.

Figure 12B presents the results of transient expression assays following particle bombardment of rice immature endosperms, where O2 and/or PBF were either: (1) independently expressed under the control of the (ubiquitin) *Ubi* promoter, *Ubi:O2* and *Ubi*:PBF, respectively

or (2) the *Ubi:O2* and *Ubi*:PBF constructs were individually co-bombarded with Glb/GUS/NOS, RP6/GUS/NOS, PG5a/GUS/NOS or Bx7/GUS/NOS. All results are given relative to the GUS/LUX ratio of the Glb/GUS/NOS, *RP6*/GUS/NOS, PG5a/GUS/NOS or Bx7/GUS/NOS construct, respectively. Error bars represent the standard deviation of the mean value from at least five independent particle bombardments.

Figure 13A is a schematic diagram of a reporter (Gt1/GUS/NOS) and effector (*Ubi:O2* and *Ubi*:PBF) constructs used in transient expression assays.

Figure 13B presents the results of transient expression assays following particle bombardment of rice immature endosperms where O2 and PBF were either independently expressed under the control of the (ubiquitin) *Ubi* promoter, *Ubi:O2* or *Ubi*:PBF, respectively; or the *Ubi:O2* and *Ubi*:PBF constructs were individually co-bombarded with Gt1/GUS/NOS. All results are given relative to the GUS/LUX ratio of Gt1/GUS/NOS construct. Error bar represents the standard deviation of the mean value for at least five independent particle bombardments.

Figure 14A is a schematic diagram of the reporter (Gt1/GUS/NOS) and effector (*35S:O2*, *35S*:PBF, *35S*: o2-676 and *35S*:PBFm) constructs used in the transient expression assay.

Figure 14B presents the results of transient expression assays following particle bombardment of rice immature endosperms, with O2, PBF, o2-676 and PBFm expressed under the control of the 35S promoter (*35S:O2*), (*35S*:PBF); (*35S*: o2-676); and (*35S*:PBFm), respectively. In addition, the activation based on co-bombard of the combination of O2 plus PBF with Gt1/GUS/NOS was evaluated, as was the combination of o2-676 plus PBFm with Gt1/GUS/NOS. The pAHC18 (Ubi/LUC/NOS) plasmid was used as an internal control for all experiments. All results are given relative to the GUS/LUX ratio of Gt1/GUS/NOS construct. Error bar represents the standard deviation of the mean value for at least five independent particle bombardments.

Figures 15A and B provide a schematic depiction of the map of 2 plasmids comprising heterologous protein coding sequences under the control of the rice endosperm-specific globulin promoter (Glb), including the Glb signal peptide, where (A) contains the lactoferrin coding sequence, and (B) contains the human lysozyme coding sequence.

Figure 16 depicts the results of a PCR analysis of T_0 transgenic plants containing Reb and the human lysozyme gene. Figure 16A is a diagram that shows the construct API266 (native-Reb), the primer positions and the size of the 522 bp amplified fragment, where one primer was designed based on the vector sequence and the other using the Reb terminator.

Figure 16B (Reb) is a diagram that shows the construct API264 (Glb-lys), the primer positions and the size of the 278 bp amplified fragment. The primers hybridize to an internal sequence of the human lysozyme gene.

Figure 16C presents the results of PCR analysis of native-Reb/Glb-Lys co-transformed plants where arrows mark the 522 bp fragment of the Reb/vector region and the 278 bp fragment derived from the internal sequence of the human lysozyme gene.

Figure 17 illustrates the results of an analysis for the expression of human lysozyme in mature seed of T_0 transgenic plants derived from progenitor cells transformed with constructs containing the human lysozyme gene expressed under the control of the Glb promoter and the Reb gene expressed under the control of its own promoter. Seeds of ten plants containing the Reb and lysozyme genes and seeds from 17 plants containing only the lysozyme gene were analyzed for lysozyme, with twenty individual seeds analyzed from each plant.

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, all technical and scientific terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook *et al.,* 1989 and Ausubel FM *et al.,* 1993, for definitions and terms of the art. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

All publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention.

An "isolated polynucleotide" or an "isolated DNA segment" having a sequence which encodes a plant transcription factor is a polynucleotide which contains the coding sequence of the plant transcription factor (i) in isolation, (ii) in combination with additional coding sequences, such as fusion protein or signal peptide, in which the plant transcription factor coding sequence is the dominant coding sequence, (iii) in combination with non-coding sequences, such as control elements, such as promoter and terminator elements, effective for expression of the coding sequence in plant cells, and/or (iv) in a vector or host environment in which the plant transcription factor coding sequence is a heterologous gene.

As used herein, the term "plasmid" refers to a circular double-stranded (ds) DNA construct used as a cloning vector, and which forms an extrachromosomal self-replicating genetic element in many bacteria and some eukaryotes.

As used herein, the term "vector" refers to a nucleic acid construct designed for transfer between different host cells. An "expression vector" refers to a vector that has the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

As used herein, the term "promoter" refers to a nucleic acid sequence that functions to direct transcription of a distal gene. The promoter will generally be appropriate to the host cell in which the target gene is being expressed. The promoter together with other transcriptional and translational regulatory nucleic acid sequences (also termed "control sequences") is necessary to express a given gene. In general, the transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

A nucleic acid sequence is "heterologous" with respect to a control sequence (*i.e.* promoter or enhancer) when it does not function in nature to regulate the same gene the expression of which it is currently regulating. Generally, heterologous nucleic acid constructs are introduced into the cell or part of the genome in which they are present, and have been added to the cell, by transfection, microinjection, electroporation, or the like. The sequences may contain a control sequence/DNA coding sequence combination that is the same as, or different from a control sequence/DNA coding sequence combination found in the native plant.

As used herein, the term "operably linked" relative to a recombinant DNA construct or vector means nucleotide components of the recombinant DNA construct or vector are in a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

As used herein, the term "gene" means the segment of DNA involved in producing a polypeptide chain, which may or may not include regions preceding and following the coding region, *e.g.* 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

The term "gene", may be used interchangeably herein with the term "nucleic acid coding sequence", and the term "structural gene" which means a DNA coding region.

As used herein, the term "fragment," when referring to a gene sequence means a polynucleotide having a nucleic acid sequence which is the same as part of, but not all of, the nucleic acid sequence of the full length gene. The fragment preferably includes at least 15 contiguous bases of the gene, preferably at least 20-30 bases. With reference to interaction with a transcription factor, the sequence must be of sufficient length to interact with the transcription factor.

As used herein, the terms "transformed", "stably transformed" or "transgenic" with reference to a plant cell means the plant cell has a non-native (heterologous) nucleic acid sequence integrated into its genome which is maintained through one or more generations.

As used herein, the term "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process generally includes both transcription and translation.

The term "introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell where the nucleic acid sequence may be incorporated into the genome of the cell (for example, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (for example, transfected mRNA).

As used herein, the term "effector", refers to plant transcription factors that "effect" the transcription of genes having the appropriate response sequence.

As used herein, the term "promoter" refers to a sequence of DNA that functions to direct transcription of a gene which is operably linked thereto. A promoter may or may not include additional control sequences (also termed "transcriptional and translational regulatory sequences"), involved in expression of a given gene product. In general, transcriptional and translational regulatory sequences include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. The promoter may be homologous or heterologous to the cell in which it is found.

As used herein, the terms "regulatable promoter" and "inducible promoter" may be used interchangeably and refer to any promoter the activity of which is affected by a cis or trans acting factor.

As used herein, the terms "transcriptional regulatory protein", "transcriptional regulatory factor" and "transcription factor" may be used interchangeably and refer to a cytoplasmic or nuclear protein that binds a DNA response element and thereby transcriptionally regulates the expression of an associated gene or genes. Transcription factors generally bind directly to a DNA response sequence or element, however in some cases may bind indirectly to the another protein, which in turn binds to or is bound to the DNA response element.

As used herein, the terms "response sequence" and "response element" refer to the binding site or sequence for a transcriptional regulatory protein (transcription factor) which may be the part of, overlapping, or adjacent to, a promoter sequence.

As used herein, a "plant cell" refers to any cell derived from a plant, including undifferentiated tissue (*e.g.*, callus) as well as plant seeds, pollen, progagules and embryos.

As used herein, the term "mature plant" refers to a fully differentiated plant.

As used herein, the terms "native" and "wild-type" relative to a given plant trait or phenotype refers to the form in which that trait or phenotype is found in the same variety of plant in nature.

As used herein, the term "plant" includes reference to whole plants, plant organs (for example, leaves, stems, roots, etc.), seeds, and plant cells and their progeny. Plant cell, as used herein includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves roots shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants which can be used in the methods of the present invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledenous and dicotyledenous plants.

As used herein, the term "transgenic plant" refers to a plant comprising within its genome a heterologous DNA segment. Generally, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic.

II. Methods and Compositions of the Invention

The invention provides transgenic plant cells and transgenic plants which express one or more recombinant transcription factors, wherein expression of the one or more transcription factors is correlated with increased expression of a gene under the control of a promoter with which one or more of the transcription factors interacts.

In activating transcription of a nucleic acid coding sequence, the recombinant transcription factors described herein may interact with (1) a native seed-specific promoter or (2) a non-native, recombinant or heterologous seed-specific promoter. In either case, all or part of the seed-specific promoter sequence is operably linked to a native nucleic acid coding sequence or a heterologous nucleic acid coding sequence (*e.g.*, a transgene) and may be from the same or a different species from that of the plant in which it is present. The transgene may be a reporter gene, such as luciferase or GUS, or a gene encoding a recombinant protein that is expressed in the plant.

In one preferred approach, temporal expression of the recombinant protein takes place and is detected in particular seed tissues due to expression under the control of a promoter which directs tissue-preferential or tissue-specific expression. See Figure 1A which illustrates various

tissues of a cereal seed, Figure 1B which outlines the life cycle of a cereal seed and Figures 1C-E which illustrate tissue-specific expression in the endosperm, embryo and alleurone tissues.

In practicing the invention, a plant cell may be transformed with one or more vectors, each comprising the coding sequence for one or more plant transcription factors, each operably linked to a tissue specific promoter, wherein the tissue specific promoters may be the same or different. It will be understood by those of skill in the art that once expressed a recombinant transcription factor may act on the promoter which is regulating expression of the transcription factor itself in addition to acting on one or more other promoters.

In one preferred embodiment, the plant transcription factor is expressed under the control of a seed specific promoter, which generally promotes selective expression in the endosperm, alleurone or embryo of a seed. In some cases, the transcription factor is expressed under the control of two or more different seed tissue-specific promoters at the same time in the same plant.

In a related embodiment, two or more transcription factors are expressed in the same cell and act in concert (in an additive, synergistic or inhibitory manner) to modulate expression of the gene to which they are operably linked. For example, when the opaque 2 (O2) and prolamin box binding factor (PBF) transcription factors are co-expressed, transcription is activated in an additive manner. (See, *e.g.*, Figs. 12B, 13B and 14B.)

In addition, when two or more transcription factors are expressed in the same cell, one transcription factor may activate expression of the other transcription factor.

In another preferred embodiment, a heterologous nucleic acid coding sequence for a first transcription factor under the control of a first seed specific promoter, and a heterologous nucleic acid coding sequence for a second transcription factor under the control of a second seed-specific promoter, are introduced into a plant cell. In such cases, the nucleic acid construct comprising the second transcription factor and the second seed specific promoter, may be in the same or a different vector from the nucleic acid construct comprising the first transcription factor and the first seed specific promoter.

It is preferred that expression of two or more transcription factors in the same plant results in a level of transgene expression which is greater than the expression of each transcription factor alone, when the transgene is under the control of a promoter with which the transcription factors interact. In other words, as exemplified herein, the level of expression observed when a transgene is expressed under the control of a promoter with which both the O2 and PBF transcription factors interact is greater than that the expression level observed due to either transcription factor alone. In this case the observed effect was additive, as shown in Figs. 12B, 13B and 14B. It is within the scope of the present invention that any combination of two or more plant transcription factors be expressed in the same plant at the same time and result in a

level of transgene expression that is additive or greater, when the transgene is under the control of a promoter with which the transcription factors interact.

In some cases a native promoter is non-responsive to a particular transcription factor. The present invention provides a method for modification of such a promoter to contain a response sequence or element with which the transcription factor may interact, as exemplified herein by the modification of the rice glutelin-1 (*Gt-1*) promoter to contain a 98 bp Reb upstream activation sequence (UAS fragment containing 3 copies of GCCACGT(C/A)AG amplified from the Glb promoter) inserted at position -630 bp distal to the TATA box of the Gt1 promoter in order to generate Gt1+UAS-GUS. The invention further provides seed specific promoters that have been modified to include the response sequence for a transcription factor not found in the native form of the promoter, wherein the modified seed specific promoter may be activated interaction with the transcription factor.

Transgenic plant cells transformed with a selected heterologous protein coding sequence under the control of a seed-specific promoter that is responsive to one or more transcription factors expressed in the cell are also provided by the invention. In some cases, a heterologous protein coding sequence is expressed under the control of two or more different seed-specific promoters at the same time in the same plant cell.

In one preferred aspect, the present invention provides transgenic plants wherein a plant transcription factor which interacts with one or more seed specific promoters is expressed in a monocot plant, resulting in a favorable modification in the production of native proteins expressed under the control of the seed specific promoter. For example, a transgenic plant may be constructed comprising the heterologous coding sequence for the O2 transcription factor, which upon expression interacts with any of a number of native seed specific promoters in the plant (*e.g.*, a Glb promoter or a Bx7 promoter), resulting in enhanced expression of genes operably linked to the O2 responsive promoters.

In another preferred aspect, the invention provides methods for producing transgenic plant cells and transgenic plants that express a selected heterologous protein coding sequence under the control of a seed-specific promoter. In one approach, such plants are obtained by co-transformation of plant progenitor cells with one or more expression vectors effective to express (1) one or more plant transcription factors, and (2) a heterologous protein coding sequence under the control of one or more seed-specific promoters responsive to the one or more plant transcription factors. Following transformation, transformant are selected and regenerated to produce transgenic plants.

In another approach, transgenic plant lines, *e.g.*, rice, wheat or barely, are developed and genetic crosses carried out using conventional plant breeding techniques. In one exemplary approach, a first stable transgenic plant line is generated where the plants express a transcription

factor, *e.g.*, O2, PBF or Reb, under the control of a seed-specific promoter. A number of such lines may be generated with varying levels of transcription factor expression. In practicing the method, these plants are crossed with a parental transgenic rice, corn, barley or wheat line that expresses a heterologous protein coding sequence (*e.g.*, a recombinant protein) under the control of a seed-specific promoter that is responsive to the transcription factor expressed in the first plant line. Plants derived from the resulting cross (F2) have a higher expression level of the heterologous protein in one or more particular seed tissues, than a corresponding non-transgenic plant.

III. Plant Transcription Factors

Transcription factors are capable of sequence-specific interaction with a gene sequence or gene regulatory sequence. The interaction may be direct sequence-specific binding in that the transcription factor directly contacts the gene or gene regulatory sequence or indirect sequence-specific binding mediated by interaction of the transcription factor with other proteins. In some cases, the binding and/or effect of a transcription factor is influenced (in an additive, synergistic or inhibitory manner) by another transcription factor.

The gene or gene regulatory region and transcription factor may be derived from the same type of plant (*e.g.*, the same species or genus) or a different type of plant.

The binding of a transcription factor to a gene sequence or gene regulatory sequence may be evaluated by a number of assays routinely employed by those of skill in the art, for example, sequence-specific binding may be evaluated directly using a label or through gel shift analysis.

The present invention involves the use of the maize *Opaque 2* (O2) and prolamin box binding factor (PBF) together with the rice Reb protein as transcriptional activators of monocot storage protein genes.

A. Opaque 2

Zeins are the prolamin class of seed storage proteins that accumulate to high levels in mature seeds of maize (Mertz *et al.*, 1964; Murphy *et al.*, 1971; Lee *et al.*, 1976). Several mutations are known to affect the accumulation of 22- and 19-kD zein proteins (reviewed in Motto *et al.*, 1989). One of these mutations, *Opaque 2* (O2), causes a severe reduction in the levels of zein gene transcripts encoding polypeptides of the 22-kD size class (Pedersen *et al.*, 1980; Burr *et al.*, 1982; Langridge *et al.*, 1982). This reduction in zein protein accumulation causes the affected kernels to take on the characteristic "opaque" appearance that distinguishes the O2 phenotype from the normally translucent, vitreous endosperm of wild-type seed.

The O2 gene has been cloned (Schmidt *et al.*, 1987; Motto *et al.*, 1988). The subsequent isolation and sequencing of the O2 cDNA (Hartings *et al.*, 1989; Schmidt *et al.*, 1990) indicated

that the O2 locus gene product contains a leucine-zipper DNA binding motif (Landschulz *et al.*, 1988), which consists of a heptameric repeat of leucines (the "zipper"), responsible for dimer formation, adjacent to a cluster of positively charged amino acids (the "basic" motif), which are responsible for sequence-specific recognition of the target DNA (Neuberg *et al.*, 1989; Turner *et al.*, 1989). Proteins such as O2 that have a basic domain followed by a leucine-zipper DNA binding motif have been designated "bZIP proteins" (Vinson *et al.*, 1989).

It has previously been shown that O2 is capable of binding to the promoter of 22-kD zein genes (Schmidt *et al.*, 1990) and that the bZIP domain in O2 mediates this binding (Aukerman *et al.*, 1991). DNA footprinting was used to identify the O2 binding site as 5'-TCCACGTAGA-3' (designated the "O2 box"). The site is located in the -300 region relative to the translation start and lies about 20 bp downstream of the highly conserved zein gene sequence motif known as the "prolamin box".

The results of gel shift assays using the O2 protein and extracts of endosperm indicate binding to the O2 target sequence. Additional studies have shown that O2 activates transcription from promoters derived from closely related zein genes when the promoters contain the O2 target sequence (Schmidt R *et al.*, 1992). Gel mobility shift assays have indicated that O2 can bind partial "O2 box" sequences and that only the ACGT core sequence may be required for some degree of binding. However, a single base substitution in the O2 target site has been shown to be sufficient to inhibit DNA binding (Schmidt R *et al.*, 1992).

A comparison of the O2 target sequence to upstream regions of the rice Glb promoter sequence indicates at least 3 potential O2 binding sites found in the -210 region (CCACGTA), the -220 region (CCACGT), and the -160 region (CCACGTA) (Fig. 9.) Similar sites have been identified in the wheat Bx7 promoter, shown in Fig. 10.

The nucleic acid sequence for the Opaque2 transcription factor may be found for example at GenBank Accession Nos. X15544 (opaque2 gene) and M29411 (opaque2 cDNA). In experiments carried out to demonstrate the present invention, the Opaque2 transcription factor coding sequence was cloned into expression vectors under the control of the rice glutelin-1 (Gt-1) and the maize ubiquitin promoters (Example 3).

The transient expression of O2 was shown to enhance the expression of GUS under the control of the rice Gt1, Globulin and wheat Bx7 promoters by approximately 10-fold (Example 3).

Further studies with the O2 protein suggested that O2 interacts *in vivo* with other endosperm proteins to bind zein gene promoters. A highly conserved 7-bp sequence (TGTAAAG) referred to as the Prolamin box (P-box) *cis*-element lies just 20 nucleotides upstream of the O2-box. This 7-bp sequence is referred to as the Prolamin box (P-box) due to its presence in the prolamins of corn, barley, wheat, oats, and sorghum (Forde *et al.*, 1985).

B. Prolamin Box Binding Factor (PBF)

In general, in cereals, nitrogen and sulfur are stored mainly in a group of proteins called the prolamins, which are synthesized only in the endosperm under tissue-specific and temporal transcriptional control. The barley prolamins, called hordeins, are structurally similar to the prolamins from other grass species and it has been observed that putative regulatory elements in the barley hordein gene promoters are conserved (Forde B.G. *et al.*, 1985; Kreis M. *et al.*, 1985).

The P-box has been shown to be conserved in both its nucleotide sequence and in its position (about -300 bp) relative to the translation initiation codon of zein and other prolamin genes [VicenteCarbajosa, 1997; Müller *et al.,* 1995; Albani *et al.*, 1997; Forde *et al.*, 1985; Hammond-Kosack *et al.*, 1993].

A protein, named prolamin-box binding factor or PBF, was cloned in maize and found to bind to the P-box. PBF was shown to contain the highly conserved CYS2-CYS2 zinc finger motif characteristic of the DOF (DNA binding with One Finger) class of DNA-binding proteins (reviewed by Yanagisawa, 1996).

A barley PBF homologue, designated as BPBF (Barley Prolamin-Box Binding Factor) was isolated from a barley endosperm library (Montana Menal *et al.*, 1998). An exemplary barley PBF (BPBF) coding sequence may be found at GenBank Accession Number AJ000991 (barley cDNA). The rice PBF and maize PBF coding sequences may be found for example at GenBank Accession numbers D11385 (rice cDNA) and ZMU82230 (maize cDNA). The rice PBF and maize PBF coding sequences were cloned into an expression vector under the control of the rice glutelin-1 (Gt-1) promoter (Fig. 11A, barley; Fig. 11B, maize).

The promoter regions from the rice glutelin genes *Gt1*, *Gt3*, *GluB-1* and *GluB-2*; the rice prolamin genes *RP6* and *PG5a*; the rice globulin gene *Glb* and the wheat glutelin gene, *Bx7* were PCR amplified and cloned into the GUS reporter cassette of pBI221, as detailed in Example 3.

Transcription factor ("effector") plasmids were prepared with the O2 and PBF coding sequences under the control of the CaMV 35S promoter, the maize ubiquitin or the rice glutelin *Gt1* promoter (SEQ ID NO:26). Transient assays using rice endosperm were carried out as described in Example 2, to evaluate the effector activity of the O2 and PBF transcription factors on GUS expression of heterologous nucleic acid constructs. Co-transfection with heterologous nucleic acid constructs comprising O2 and PBF, respectively, resulted in an additive increase in transactivation of the promoters from rice storage protein genes including *Gt1*, *Gt3*, *GluB-1* and *GluB-2*; the rice prolamin genes *RP6* and *PG5a*; the rice globulin gene *Glb* and the wheat glutelin gene, *Bx*, as summarized in Table 2, suggesting that the maize O2 and PBF proteins can act singly or additively as effective stimulators of heterologous storage protein promoters in developing rice seed (See Example 3.). An additive increase in transactivation was observed by co-bombardment of both effector plasmids and was observed in all the promoters of storage

protein genes that were tested, while the responsiveness of non-storage protein genes like rice actin and *CaMV 35S* to O2 and PBF was insignificant (Example 3).

When assayed in developing rice endosperm cells, both O2 and PBF were shown to individually increase transcription of a GUS reporter under the control of the rice glutelin gene, *Gt1*, however, mutant forms of O2 and PBF, defective in DNA binding, did not function as transcriptional activators. In addition, co-bombardment of Gt1/GUS/NOS with plasmids expressing the DNA binding domains for O2 and PBF in antisense orientation resulted in a expression of Gt1/GUS/NOS that was below background levels.

C. Reb

In cereals bZIP proteins have been identified as transcriptional factors for genes encoding storage proteins in the endosperm [VicenteCarbajosa, 1998; Schmidt, 1992; Conlan, 1999; Holdsworth, 1995; Liu, 1998; Mena, 1998; Onate, 1999; Schwechheimer, 1998; Schwechheimer, 1998; Wang, 1998; Yunes, 1994]. The basic amino acid containing domain of bZIP proteins has been demonstrated to bind to a recognition nucleotide sequence in the promoter, while their leucine repeat domain interacts with the transcriptional machinery leading to a dramatic increase in transcription initiation of the storage protein-encoding gene in the endosperm of the cereal grain. [See, *e.g.*, Mena, 1998 and Schmidt, 1992].

Nakase *et al.*, 1997, have described a cloned bZIP protein gene from rice, named Reb for rice endosperm bZIP protein. Reb was demonstrated to bind specifically to the sequences GCCACGTAAG and GCCACGTCAG in the distal part of the rice globulin (Glb) gene promoter, however, its function as a transcriptional activator or suppressor was not described. The complete coding sequence for Reb, isolated from rice endosperm may be found at GenBank Accession number ABO21736. (Nakase *et al.*, 1997.)

Reb was cloned from a rice BAC library and the function of the Reb gene explored. Figures 2A-I present a double-stranded depiction of the DNA sequence of the rice (*Oryza sativa*) Reb bZIP protein. The Reb gene sequence of 6.227 kb consists of 5 introns and 6 exons flanked by 1.2kb of the 5' promoter and 1.2 kb of the 3' region (Fig. 3). Effector constructs containing the Reb gene together with the native Reb promoter and fusion genes linking Reb to the globulin (Glb) gene promoter, the rice actin (Act) promoter or the native Reb promoter were prepared and used to identify Reb as a transcriptional activator. (See Figs. 5A-C.)

Individual plasmids carrying the rice globulin (Glb) gene promoter fused to the GUS reporter gene and the rice glutelin 1 (Gt-1) gene promoter fused to the GUS reporter gene, respectively were constructed using standard molecular biological techniques and used for transient expression assays, as detailed in Example 2.

The endosperms of rice spikelets (7-9 days after pollination, DAP) of M2O2 (*Oryza sativa Japonica subsp.*) collected from greenhouse grown plants were bombarded with gold particles coated with DNA consisting of a mixture of the reporter gene, effector gene, and an internal control gene, typically at a molar ratio of 1:1:1. After bombardment, the immature endosperms were incubated, harvested and analyzed for GUS expression.

The results shown in Fig. 6B indicate that GUS expression was increased when Reb was expressed under the control of the globulin promoter, the actin promoter or the native Reb promoter (Figs. 5A-C) suggesting that Reb is effective to an activate expression mediated by Glb promoter.

The upstream activation sequence (UAS) for Reb was identified using a band-shift assay (Nakase *et al.*, 1997). The Glb promoter sequence which contains the UAS and the Gt-1 promoter which does not, were used to demonstrate transcriptional activation by Reb through the UAS by loss-of-function and gain-of-function experiments (Example 2).

The results described in Examples 1 and 2 show that (1) Reb is a transcriptional activator; (2) Reb specifically activates the Glb promoter but not gluletin (*Gt-1*) gene family promoters; and (3) Reb interacts with an approximately 100 bp upstream activation sequence (UAS) containing the motifs GCCACGTCAG and GCCACGTAAG (GCCACGT(A/C)AG) of the Glb promoter, as confirmed by loss-of-function and gain-of-function experiments.

IV. Heterologous Nucleic Acid Constructs

A. Constructs for Expression of a Transcription Factor in a Plant Cell

A heterologous nucleic acid construct or expression vector designed for operation in plants comprising the coding sequence for a plant transcription factor may be used to transiently or stably transform a plant, *e.g.* a monocot plant. An exemplary heterologous nucleic acid construct or expression vector designed for operation in plants, includes (i) a promoter (transcriptional regulatory region) induced in particular seed tissue ("seed-specific"), (ii) the coding sequence for a plant transcription factor operably linked to the promoter, (iii) companion sequences upstream and downstream which are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move the DNA from bacteria to the desired plant host; (iv) a selectable marker sequence; and (v) a transcriptional termination region generally at the opposite end of the vector from the transcription initiation regulatory region. Suitable transformation vectors for the preparation of such constructs are known in the art and many are commercially available.

Vector components may also include a signal sequence. The desired recombinant protein or polypeptide may be produced directly, or as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site

at the N-terminus of the mature protein or polypeptide. Included in heterologous nucleic acid constructs for use in the methods of the invention are signal sequences that allow processing and translocation of the protein, as appropriate. The heterologous nucleic acid construct typically lacks any sequence that might result in the binding of the desired protein to a membrane.

In some cases, the recombinant protein may be produced as a precursor protein, which may be further processed in the plant cell culture or following extraction from the plant.

B. Expression Vector Components

1. Seed-specific Promoters

The transcription regulatory or promoter region of the chimeric gene or heterologous nucleic acid construct is preferably a seed-specific promoter, for example, a promoter capable of directing expression of a gene product under its control, which is specific to the seed embryo, alleurone, outer layer of the endosperm or center of the endosperm; or a promoter capable of directing expression of a gene product under its control, which is specific to starch or protein synthesis.

Exemplary preferred promoters include a glutelin (Gt-1) promoter which effects gene expression in the outer layer of the endosperm and a globulin (Glb) promoter which effects gene expression in the center of the endosperm. Promoter sequences for regulating transcription of operably linked coding sequences include naturally-occurring promoters, or regions thereof capable of directing seed-specific transcription, and hybrid promoters, which combine elements of more than one promoter. Methods for construction of hybrid promoters are well known in the art.

In some cases, the promoter is derived from the same plant species as the plant in which the nucleic acid construct is to be introduced. Promoters for use in the invention are typically derived from cereals such as rice, barley, wheat, oat, rye, millet, triticale or sorghum.

Alternatively, a seed-specific promoter from one type of monocot may be used regulate transcription of a gene coding sequence from a different monocot or a non-cereal monocot. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of plant host cells. In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences.

Effective seed-inducible or seed-regulated transcriptional initiation regions (*e.g.*, promoters) may be isolated from various seed tissues and/or at various stages of seed development by a variety of techniques routinely used by those of skill in the art, including, but not limited to: (1) conventional hybridization techniques using known coding sequences from a

different species, tissue and/or developmental stage, followed by walking upstream to identify the associated transcriptional initiation sequence; (2) subtractive hybridization (Lee, *et al.*, 1991), (3) differential display (Liang *et al.,* 1997), and (4) selective amplification via biotin- and restriction-mediated enrichment, SABRE (Lavery *et al.*, 1997).

Promoters from seed tissue specific genes such as those described in Müller *et al.*, 1993, are suitable for use in the chimeric gene constructs described herein. More specifically, representative seed-associated promoters for use in the invention include the promoters from the rice glutelin multigene family, Gt1, Gt2, Gt3, GluA-3, and GluB-1. Promoter regions for these genes are described, for example, in Takaiwa, *et al.*, 1987 (rice glutelin gene, GenBank Accession Nos. D26365 and D26364), Takaiwa, *et al.*, 1991 (rice GluA-3 gene, GenBank Accession No. X54313), Takaiwa, *et al.*, 1991a (rice glutelin gene, GenBank Accession No. Y00687), Takaiwa, *et al*, 1991b (rice Glu-B gene, GenBank Accession No. X54193; rice GluB-2 gene, GenBank Accession No. X54192); rice GluB-1 gene (GenBank Accession No. X54314); Okita, *et al.*, 1989 (rice Gt2 gene, GenBank Accession No. L36819 M28157); rice Gt3 gene, (GenBank Accession No. M28158); rice Gt1 gene (GenBank Accession No. M28156); rice glutelin gene (GenBank Accession Nos. D26363, D26366, D26367, D26368 and D26369); Abe, *et al.*, 1989 (rice prepro-glutelin gene, GenBank Accession No. D00584); Kim and Wu, 1990 (rice glutelin gene, GenBank Accession No. X52153). In general, these promoters are active during seed development and direct endosperm-specific expression (Takaiwa *et al.*, 1991a, 1991b; Okita *et al.*, 1989; Abe *et al.*, 1989; Kim and Wu, 1990).

Other suitable seed-associated promoters include the promoter regions from the rice prolamin gene (GenBank Accession No. D73384; Nakase *et al.*, 1996); the barley B22EL8 gene promoter, which directs expression in immature aleurone layers (Klemsdal *et al.*, 1991); the promoter for the barley LTp gene (GenBank Accession No. X57270); the barley β-amylase (Kreis *et al.*, 1987; GenBank Accession No. X52321 and M36599) and β-glucanase gene promoters (Wolf 1992) *e.g.*, the barley G1b gene promoter (GenBank Accession No. X56775); the barley CMd gene promoter (Halford *et al.*, 1988; GenBank Accession No. X13198), and promoters from the barley hordein gene family of seed storage proteins, *e.g.*, B-, C-, and D-hordein genes (Sorensen, *et al.*, 1996 and references therein), Brandt, *et al.*, 1985 (hordein B1 gene promoter, GenBank Accession No. X87232), Entwistle, 1988 (barley hordein C promoter, GenBank Accession No. M36941), and Müller *et al.,* 1993 (barley hor1-17 gene, GenBank Accession No. X60037). Hordein gene promoters such as the Hor3 gene promoter (Sorensen *et al.*, 1996; GenBank Accession No. X84368) direct the specific expression of the corresponding genes in the endosperm. Additional seed-induced promoters for use in the invention are the maize zein gene promoter and promoters from wheat glutenin genes. Representative wheat

glutenin gene sequences as sources for promoters for use in practicing the present invention include GenBank Accession Nos. U86O28, U86O29, and U86030.

Seed-associated corn promoters also find use in the present invention. For example, the corn *O2*-opaque 2 gene promoter (Schmidt *et al.*, 1987, GenBank Accession No. M29411); the corn *Sh2*-shrunken 2 gene promoter (Shaw *et al.*, 1994, GenBank Accession No. S48563); the *Bt2*-brittle 2 gene promoter; and the *Zp1* zein gene promoter (Burr *et al.*, 1982), all of which induce endosperm-specific expression. Additional examples include the *Agp1* and *Agp2* gene promoters (Giroux *et al.*, 1994), which are embryo-specific promoters. The sequences of the above-described promoters, and/or the structural sequences from which such promoters may obtained, are expressly incorporated by reference herein.

Any of the above promoters may also be obtained from an alternative monocot species. For example, a promoter such as the Gt1 gene promoter from rice may be isolated from other cereal-derived nucleic acid containing extracts, *e.g.*, wheat, oat, or the like, using conventional hybridization techniques known in the art.

2. Coding Sequence

The heterologous nucleic acid constructs described herein may comprise the coding sequence for a plant transcription factor, as further described above for O2, PBF, BPBF and Reb. (See, also Example 1.)

Alternatively, the heterologous nucleic acid constructs described herein may comprise a heterologous protein or transgene coding sequence. Exemplary heterologous protein coding sequences include, but are not limited to, the coding sequence for a human milk protein *e.g.*, lactoferrin or lysozyme.

3. Expression Vector Components

Expression vectors or heterologous nucleic acid constructs, designed for operation in plants, comprise companion sequences upstream and downstream from the expression cassette. The companion sequences are of plasmid or viral origin and provide necessary characteristics to the vector to permit the vector to move DNA from bacteria to the plant host, such as, sequences containing an origin of replication and a selectable marker. Typical secondary hosts include bacteria and yeast.

In one embodiment, the secondary host is *E. coli*, the origin of replication is a colE1-type, and the selectable marker is a gene encoding ampicillin resistance. Such sequences are well known in the art and are commercially available as well (*e.g.*, Clontech, Palo Alto, Calif.; Stratagene, La Jolla, CA).

The transcriptional termination region may be taken from a gene where it is normally associated with the transcriptional initiation region or may be taken from a different gene. Exemplary transcriptional termination regions include the NOS terminator from *Agrobacterium* Ti plasmid and the rice α-amylase gene terminator.

Polyadenylation signals (Alber *et al.*, 1982) may also be added to the expression cassette to optimize high levels of transcription and proper transcription termination, respectively. Polyadenylation sequences include but are not limited to the *Agrobacterium* octopine synthetase signal (Gielen *et al.*,1984) or the nopaline synthase of the same species (Depicker *et al.*, 1982).

Suitable selectable markers for selection in plant cells include, but are not limited to, antibiotic resistance genes, such as, kanamycin (*npt*II), G418, bleomycin, hygromycin, chloramphenicol, ampicillin, tetracycline, and the like. Additional selectable markers include a bar gene which codes for bialaphos resistance; a mutant EPSP synthase gene which encodes glyphosate resistance; a nitrilase gene which confers resistance to bromoxynil; a mutant acetolactate synthase gene (ALS) which confers imidazolinone or sulphonylurea resistance; and a methotrexate resistant DHFR gene.

The particular marker gene employed is one that allows for selection of transformed cells as compared to cells lacking the DNA that has been introduced. Preferably, the selectable marker gene is one that facilitates selection at the tissue culture stage, *e.g.*, a kanamycin, hygromycin or ampicillin resistance gene.

The vectors of the present invention may also be modified to intermediate plant transformation plasmids that contain a region of homology to an *Agrobacterium tumefaciens* vector, a T-DNA border region from *Agrobacterium tumefaciens*, and chimeric genes or expression cassettes (described above). Further, the vectors of the invention may comprise a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*.

In general, a selected nucleic acid sequence is inserted into an appropriate restriction endonuclease site or sites in the vector. Standard methods for cutting, ligating and *E. coli* transformation, known to those of skill in the art, are used in constructing vectors for use in the present invention. Generally, vectors for use in practicing the present invention are constructed using methods known to those skilled in the art. See generally, Maniatis *et al.*, 1989; Ausubel *et al.*, (c) 1987, 1988, 1989, 1990, 1993, and Gelvin, S.B., *et al.*, 1990, all three of which are expressly incorporated by reference, herein.

V. Generation of Transgenic Plants

A. Plants

The plants used in practicing the invention are generally of monocot origin, particularly the members of the taxonomic family known as the *Gramineae*. This family includes all members of the grass family of which the edible varieties are known as cereals or grains. The cereals include a wide variety of species such as wheat (*Triticum sps.*), rice (*Oryza sps.*), barley (*Hordeum sps.*), oats (*Avena sps.*), rye (*Secale sps.*), corn (*Zea sps.*), and millet (*Pennisettum sps.*). In one embodiment of the invention, preferred family members are rice, wheat and barley.

Plant cells or tissues derived from the members of the family may be transformed with expression vectors (*i.e.*, plasmid DNA into which the gene of interest has been inserted) using a variety of standard techniques (*e.g.*, microparticle bombardment, electroporation, protoplast fusion or infection with *Agrobacterium*).

Transgenic plant cells obtained as a result of such transformation express the coding sequence for a plant transcription factor such as Reb, O2 or PBF. The transgenic plant cells are cultured in medium containing the appropriate selection agent to identify and select for plant cells which express the heterologous nucleic acid sequence. After plant cells that express the heterologous nucleic acid sequence are selected, whole plants are regenerated from the selected transgenic plant cells. Techniques for regenerating whole plants from transformed plant cells are generally known in the art.

In one embodiment of the invention, transgenic plant lines, *e.g.*, rice, corn, wheat or barely, are developed and genetic crosses carried out using conventional plant breeding techniques. In one example of this approach, a first stable transgenic plant line is generated where the plants express a transcription factor, *e.g.*, O2, PBF or Reb, under the control of a seed-specific promoter. A number of such lines may be generated with varying levels of transcription factor expression. The plants are crossed with a second transgenic plant line that expresses a heterologous protein coding sequence (*e.g.*, a recombinant protein) under the control of a seed-specific promoter that is responsive to the transcription factor expressed in the first plant line. The resulting cross (F2) has a higher expression level of the heterologous protein in one or more particular seed tissues, dependent upon the promoter used.

B. Transformation of Plant Cells

Vectors useful in the practice of the present invention may be microinjected directly into plant cells by use of micropipettes to mechanically transfer the nucleic acid construct or cassette (Crossway, 1985). Such nucleic acid constructs or cassettes may also be transferred into the plant cell using polyethylene glycol. In addition, high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the

surface may also be used for introduction of nucleic acid sequences into plant cells. (See, *e.g.*, Klein *et al.*, 1987 and Knudsen *et al.*, 1991).

Additional methods for introduction of nucleic acid sequences into plant cells include fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible forms for introduction of nucleic acid sequences into plant cells with lipid surfaces (Fraley *et al.*, 1982); and electroporation (From *et al.*, 1985). In this technique, electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of plasmids into plant cells or protoplasts. Electroporated plant protoplasts will reform the cell wall, divide, and form plant callus.

Another preferred method of introducing a nucleic acid construct into plant cells is to infect a plant cell, explant, meristem or seed with *Agrobacterium*, in particular *Agrobacterium tumefaciens*. A nucleic acid construct comprising such a sequence of interest can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells upon infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome (Horsch *et al.*, 1984; Fraley *et al.*, 1983; Schell, 1987).

Standard *Agrobacterium* binary vectors are known to those of skill in the art and many are commercially available. Expression vectors typically include polyadenylation sites, translation regulatory sequences (*e.g.*, translation start sites), introns and splice sites, enhancer sequences (which can be inducible, tissue specific or constitutive), and may further include 5' and 3' regulatory and flanking sequences.

An exemplary binary vector suitable for use in practicing the invention include at least one T-DNA border sequence (left, right or both); restriction endonuclease sites for the addition of one or more heterologous nucleic acid coding sequences [adjacent flanking T-DNA border sequence(s)]; a heterologous nucleic acid coding sequence (*i.e.*, the sequence encoding a protein or polypeptide of interest), operably linked to appropriate regulatory sequences and to the directional T-DNA border sequences; a selectable marker-encoding nucleotide sequence which is functional in plant cells, operably linked to a promoter effective to express the selectable marker encoding sequence; a termination element for the selectable marker-encoding nucleotide sequence; a heterologous Ti-plasmid promoter; a nucleic acid sequence which facilitates replication in a secondary host (*e.g.*, an *E. coli* origin of replication) and a nucleic acid sequence for selection in the secondary host, *i.e.*, *E. coli*.

In one aspect of the invention, an *Agrobacterium* binary plant transformation vector is introduced into a disarmed strain of *A. tumefaciens* by electroporation (Nagel, *et al.*, 1990), followed by co-cultivation with plant cells, to transfer the heterologous nucleic acid construct(s)

into plant cells. Upon infection by *Agrobacterium tumefaciens*, the heterologous DNA sequence is stably integrated into the plant genome in one or more locations.

In a further aspect of the invention, transgenic plants are produced using *Agrobacterium* T-DNA vectors or microprojectile bombardment, where a heterologous nucleic acid coding sequence is integrated into the plant genome and traditional breeding is used to generate transgenic seed stock and transgenic plants.

Suitable selectable markers for selection in plant cells are described above and the particular marker gene employed is one which allows for selection of transformed cells as compared to cells lacking the DNA which has been introduced. Preferably, the selectable marker gene is one which facilitates selection at the tissue culture stage, *e.g.*, a kanamycin, hygromycin or ampicillin resistance gene.

Transformed explant cells are screened for the ability to be cultured in selective media having a threshold concentration of selective agent. Explants that can grow on the selective media are typically transferred to a fresh supply of the same media and cultured again. The explants are then cultured under regeneration conditions to produce regenerated plant shoots. After shoots form, the shoots are transferred to a selective rooting medium to provide a complete plantlet. The plantlet may then be grown to provide seed, cuttings, or the like for propagating the transformed plants. The method provides for efficient transformation of plant cells with expression of modified native or non-native plant genes and regeneration of transgenic plants, which can produce a recombinant protein or polypeptide of interest.
The expression of a recombinant protein or polypeptide can be confirmed using any of a number of standard analytical techniques such as Western blot, ELISA, PCR, HPLC, NMR, or mass spectroscopy.

VI. Utility

The results presented herein suggest that the expression of the cDNA for the maize transcription factor cDNA encoding *opaque* 2 (O2) and/or the prolamin box binding factor (PBF) find utility in the enhanced gene expression of a coding sequence under the control of a promoter with which the transcription factor interacts. An approximately 10 fold enhancement of expression is observed when O2 or PBF is expressed under the control of a rice Gt1, Globulin or wheat Bx7 promoter. The results also suggest the utility of co-expression of the O2 and PBF transacting factors for further enhancement of gene expression and that co –expression of one or more transcription factors together with a heterologous protein coding sequence under the control of a promoter responsive to the one or more transcription factors may facilitate enhanced recombinant protein expression.

Such co-expression may be accomplished by development of transgenic plant lines which express one or more transcription factors together with the heterologous protein coding sequence or by genetic crosses of plant lines which express individual transcription factors with plant lines that express the heterologous protein coding sequence.

Accordingly, the expression of specific transcription factors in transgenic plants, as described herein, provides a means to increase the expression of recombinant proteins in cereal grains.

All publications, patents and patent applications are herein expressly incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The following examples illustrate but are not intended in any way to limit the invention.

EXAMPLE 1

Plant Transcription Factors

A. Cloning of the Reb Gene from a Rice BAC Library

Reb was cloned from a rice BAC library. The Reb gene including the introns, promoter and 3'- UTR region is 6,227 bp long, comprises 6 exons and 5 introns and is flanked by a 1.2 kb 5' promoter and a 1.2 kb 3'-terminator region. The function of the Reb gene was explored using effector constructs containing the Reb gene together with the native Reb promoter and fusion genes linking Reb to the rice actin (Act) or globulin (Glb) gene promoters. (See Fig. 6A-B.)

PCR primers were designed based on the Reb gene sequence provided in Nakase, 1997 and used to screen a rice bacterial artificial chromosome (BAC) library [Yang, 1997] using a screening strategy for tri-dimensional DNA pools of the BAC library as described by Xu, 1998.

PCR was carried out with 100 ng pooled BAC DNA, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 μM dNTP and employed a program of denaturing at 94 °C for 5 min followed by 30 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 1 min, using a forward primer (5'-CTGATATGTGCCCATGTTCCAAAC-3'; SEQ ID NO:1) and a reverse primer (5'-CCTTGCTGAATGCAGATGTTTCAC -3'; SEQ ID NO:2). The plasmid DNA of a positive BAC clone was prepared as described [Yang, 1997], the BAC DNA digested with *Hind*III and the presence of the Reb gene confirmed by Southern analysis [Sambrook J., 1989].

The Reb gene was retrieved from the BAC by subcloning two fragments into the pBluescript KS+ vector (Stratagene, CA). First, the promoter and partial coding region was obtained as a *Kpn*I-*Hind*III fragment, followed by a second step where a *Hind*III fragment containing the remaining coding region and the 3' terminator region was obtained by shut-gun cloning. The two fragments were ligated at the internal *Hind*III site generating an intact Reb

gene and the complete Reb gene was generated by ligating a 1,775 bp fragment containing the promoter and the 5' coding region, to a 4,452 bp fragment containing the 3' coding and terminator region.

The Reb DNA was sequenced with an automatic DNA sequencer (ABI 371) which revealed 5 introns, 6 exons, 1.16 kb of the 5' promoter sequence and 1.2 kb of the 3' region totaling 6,227 bp. (Fig. 2A-I). A comparison of the open reading frame of the isolated Reb gene with the Reb cDNA gene found at GenBank Accession No. ABO21737 revealed 99.97% DNA sequence similarity and 99.99% amino acid similarity resulting from two amino acid changes: $Ile_{165} \rightarrow Asn$ and $Glu_{215} \rightarrow Lys$. These differences are likely to be due to polymorphisms among rice varieties.

B. Opaque2 (O2) and Prolamin Box Factor (PBF)

The nucleic acid sequence for the Opaque2 transcription factor found for example at GenBank Accession number X15544 (opaque2 gene), M29411 (opaque2 cDNA) was cloned into an expression vector under the control of the rice glutelin-1 (Gt-1) promoter (Fig. 11C, maize).

The rice PBF and maize PBF coding sequences found for example at GenBank Accession Nos. D11385 (rice cDNA) and ZMU82230 (maize cDNA) were also cloned into an expression vector under the control of the rice glutelin-1 (Gt-1) promoter.

The DNA sequence of the rice (*Oryza sativa*) globulin promoter, ("Glb") with putative binding sites for the O2 transcription factor and the prolamin box and the DNA sequence of the wheat Bx7 promoter with putative binding sites for the O2 transcription factor and the prolamin box are shown in Figs. 9 and 10, respectively. The coding sequence for the wheat Bx7 gene is presented at GenBank Accession Nos. M22209.

Promoters were digested to produce the appropriate cohesive ends and cloned into compatible sites in a reporter construct. In one example, the reporter construct is comprised of the rice globulin (Glb) or wheat Bx7 promoter translationally fused with GUS, with the resulting constructs designated, Glb/GUS/NOS and Bx7/GUS/NOS (Fig. 12A).

The effect of plant transcription factors on the gene expression was evaluated by co-transformation with a heterologous nucleic acid construct effective to express the transcription factor, *e.g.* O2, PBF, BPBF or Reb.

EXAMPLE 2

Transient Expression Assays with the Reb Transcription Factor

A. Plasmid Construction

Plasmids were constructed using standard molecular biological techniques as described in Ausubel *et al.*, 1987. Plasmids API212 (Glb-GUS) carrying the rice globulin gene promoter

fused to the GUS reporter gene and API142 (Gt1-GUS) containing the rice glutelin 1 gene promoter fused to the GUS reporter gene were used for transient expression assays. The globulin promoter (GenBank Accession number X63990) and glutelin 1 promoter (GenBank Accession number Y00687) were obtained from *M2O2* (*Oryza sativa Joponica* subsp.) by amplification with PCR. The plasmid containing the Reb gene under the control of its native promoter was designated pAPI267 (Native-Reb). The plasmid designated Glb-Reb (pAPI266) was prepared by cloning the Reb coding region (*Nru*I/*Sac*I fragment) into the Glb-GUS plasmid after removal of the GUS gene by digestion with *SmaI/SacI*, thus replacing the GUS gene with the Reb gene. Using the same strategy, the plasmid designated Actin-Reb (pAPI277) was made by replacing the GUS gene of plasmid Act1-D-GUS (McElroy, 1990) with the complete Reb gene (*NruI/SacI* fragment). The Act1-D promoter was kindly provided by Professor Ray Wu, Cornell University (McElroy, 1990).

B. Transient Assay using Rice Endosperm

Rice spikelets with immature endosperm (7-9 days after pollination, dap) of M2O2 (*Oryza sativa Japonica subsp.*) were collected from plants grown in the greenhouse at 30°C. The spikelets were sterilized with 70 % ethanol for 10 min. After evaporation of residual ethanol, the endosperm was dissected and 10 immature endosperms placed on a filter paper in a Petri dish containing AA medium [Chen *et al.*, 1998] supplemented with 20mM ammonium nitrate.

Fifty µl of gold particles (60mg·ml^{-1} at 1:1 ratio of 1.0 and 1,5-3.0 µm diameter gold particle) were coated with 5 µg DNA consisting of a mixture of the reporter gene, the effector gene, and the internal control gene, typically at a molar ratio of 1:1:1. DNA coating was accomplished as described in the instruction manual of the Biolistic PDS system (Bio-RAD, Hercules, CA, USA). pAHC18 containing the luciferase gene driven by an ubiquitin promoter [Christensen *et al.*, 1996] was used as an internal control. In tests without the effector gene, pAHC18 was replaced by pBluescript DNA. Particle bombardment was carried out with a biolistic Helium gun device at 1100 psi (Biolistic PDS 1000/He system, Bio-Rad, Hercules, CA, USA). After bombardment, the immature endosperms were incubated at 25°C for 24h in 5 ml of AA medium supplemented with 20 mM ammonium nitrate, 50µg·ml^{-1} cefotaxin and 50µg·ml^{-1} timentin (Sigma, Louis, MO, USA) to prevent bacterial growth. The endosperms were then harvested and ground with 55 µl extraction buffer (0.1 M potassium phosphate, pH 8.0, 1 mM EDTA, 10 mM DDT, 5 % glycerol, 0.2mM leupeptin and 0.2 µM phenylethylsulfonyl fluoride (PMSF). The extract was centrifuged at 25,000 g for 5 min at 4°C. From the supernatant, a 20 µl aliquot was added to 180 µl of luciferase assay buffer (0.25 M Tricine, pH 7.8, 150 mM magnesium chloride, 10 mM ATP, 1 mM DDT and 100 µg ml^{-1} BSA). Another 20 µl aliquot

was added to 200 µl of GUS assay buffer (Tropix, Bedford, MA, USA). Luciferase activity was measured after incubation at 25°C for 20 min and β-glucuronidase activity after 1h at 37°C for 1h with a Monolight 2010 chemi-illuminometer according to the manufacturer's instructions (Analytical Luminescence Lab., Monolight, San Diego, CA, USA). β-glucuronidase activity was normalized to the luciferase activity and expressed relative to the activity of the Glb-GUS. In general, the data presented reflect an average of at least six assays of two independent experiments.

C. Transcriptional Activation with Reb

The function of Reb gene was analyzed by the transient assay with rice immature endosperm. Transient expression studies were carried out to evaluate the effect of Reb on expression of GUS (*β*-glucuronidase), under the control of the Glb promoter.

The results shown in Fig. 6B indicate that GUS expression was increased when Reb was expressed under the control of the globulin promoter, the actin promoter or the native Reb promoter suggesting that Reb is effective to an activate expression mediated by Glb promoter.

Putative Reb binding sites in the globulin (Glb) promoter were identified as shown in Fig. 4.

In order to determine, if binding of the Reb protein to the motif (GCCACGT(A/C)AG) in the globulin (Glb) gene promoter activates transcription of this promoter, plasmids containing fusions of the Reb coding region with the Glb promoter and the rice actin (Act) gene promoter were prepared (Fig. 6A). These as well as the expression plasmid containing the native Reb gene (pAPI266) were co-bombarded into the rice endosperm with a plasmid containing the GUS reporter gene driven by the Glb gene promoter and an internal control plasmid containing the luciferase gene driven by the ubiquitin promoter.

Setting the level of GUS expression by the globulin gene promoter as 1, the co-delivery of the plasmids containing the Reb gene increased GUS expression irrespective of whether the gene was driven by its own promoter or the Glb promoter or the Actin promoter (Fig. 6B). The increases were 2.43, 2.01 and 1.98 fold, respectively. The activation of GUS expression was abolished when a promoter-less Reb construct was co-bombarded with Glb-GUS (Fig. 6B). These results suggested that the Reb protein functions as a transcriptional activator of the Glb promoter.

D. Identification of the Upstream Activation Sequence (UAS) for Reb

Using a band-shift assay, Nakase *et al.*, 1997 have shown that Reb binds to two motifs, GCCACGTAAG or GCCACGTCAG. An analysis of the Glb promoter sequence revealed two

copies of GCCACGTAAG and one copy of GCCACGTCAG clustered in the sequence region -700 bp distal to the TATA box of the promoter (Fig. 4).

In order to determine whether the binding motifs for Reb signify an upstream activation sequence (UAS), 200 bp of the Glb promoter, which contains the three motifs located at positions -642 to -842 distal to the TATA box were deleted from the Glb promoter (Fig. 7A). The deletion was demonstrated to have no effect on the expression of GUS as both the Glb-GUS and the GlbΔUAS-GUS (UAS deleted) constructs showed the same level of background GUS expression (Fig. 7B).

When Native-Reb was co-bombarded with GlbΔUAS-GUS, the transcriptional activation by Reb which was evident when Native-Reb was co-bombarded with Glb-GUS was lost (Fig. 7B). These data indicate the transcriptional activation of Glb-GUS by Reb occurs through this 200bp fragment containing Reb binding motifs.

A scan of the rice glutelin1 (Gt1) promoter sequence did not reveal the presence of Reb binding motifs. Accordingly, Gt1 was selected as a candidate for the introduction of the UAS from the Glb promoter in order to test for gain of the Reb response function. Heterologous nucleic acid constructs were prepared containing the native Gt1 promoter linked to the GUS gene (Gt1-GUS), and a Gt1 promoter modified to contain a 98 bp Reb UAS fragment containing 3 copies of GCCACGT(C/A)AG (amplified from the Glb promoter) was inserted at position -630 bp distal to the TATA box of the Gt1 promoter in order to generate Gt1+UAS-GUS (Fig. 8A).

When Gt1-GUS was tested by co-bombardment of developing endosperm with the native Reb gene and Gt1-GUS, the results showed that Reb does not activate the Gt1 promoter (Fig. 8B). Gt1+UAS-GUS was tested for GUS expression and it was shown that addition of the Reb UAS to the Gt1 promoter did not increase its capacity for GUS expression significantly (Fig. 8B). However, when Gt1+UAS-GUS was tested by co-bombardment of developing endosperm with the native Reb gene a 2.5 fold increase in GUS activity was obtained (Fig. 8B).

The Reb protein was previously described as a transcription factor. The results described herein show that (1) Reb is a transcriptional activator, as evidenced by a 2.0 to 2.5-fold increase in GUS activity when Reb effector constructs were co-transferred with the reporter *uid* A gene encoding GUS under the control of the Glb promoter into immature rice endosperm cells; (2) Reb specifically activates the Glb promoter but not gluletin gene family promoters; (3) Reb interacts with an approximately 100 bp upstream activation sequence (UAS) containing the motifs GCCACGTCAG and GCCACGTAAG (GCCACGT(A/C)AG) of the Glb promoter, as confirmed by loss-of-function and gain-of-function experiments. The loss of activation function, when the 200bp fragment containing the Reb UAS is removed from the Glb gene promoter, and the gain of this function, when the 98bp fragment with Reb UAS is added to the Gt1 promoter, establishes the 98bp fragment as an upstream activation sequence (UAS).

EXAMPLE 3

Transient Expression Assays with the O2 and PBF Transcription Factors

A. Plasmid Construction

Plasmids were constructed using standard molecular biological techniques as described in Ausubel *et al.*, 1987. A 693 bp segment of the *Gt1* promoter sequence was removed from pGt1 v3.0 SDM, a *Gt1* expression vector, and used to replace the *CaMV 35S* promoter in pBI221 through *Hind*III and *Sma*I sites, resulting in Gt1/GUS/NOS. The promoter regions from the rice glutelin genes *Gt3*, *GluB-1* and *GluB-2*; the rice prolamin genes *RP6* and *PG5a*; the rice globulin gene *Glb*; and the wheat glutelin gene, *Bx7;* were PCR amplified from M2O2 genomic DNA or the wheat variety, Anza, for *Bx7*.

The PCR amplifications were carried out using the GeneAmp PCR system (model 2400, Perkin-Elmer) operated according to the manufacturers instructions. Basic cycling conditions were 30 cycles, after a 2 minute pre-denaturing step at 95 °C, with a 30 second denaturing step at 95 °C, a 30 second annealing step at specific temperature, and a 2 minute extension step at 72 °C. The final extension step was 5 minute at 72 °C, followed by 4 °C soaking step. Reaction components per 50 µl volume, were 1 µg of genomic DNA or 1 ng of plasmid DNA, 2.5 µl of 5 µM primer mixture, 5 µl of 10 mM dNTP, 2.5 units of *Taq* polymerase (Perkin-Elmer), 5 µl of 10X PCR buffer (Perkin-Elmer). The concentration of $MgCl_2$ was 1.5 mM, for all the promoters with the exception of the *Bx7* promoter for which 2.5 mM $MgCl_2$ was used. All the PCR primers and amplified fragment sizes are presented in Table 1.

Each of the PCR amplified promoter sequences was cloned into the GUS cassette of pBI221 through *Pst*I and *Xba*I sites to give Gt3/GUS/NOS, GluB-1/GUS/NOS, GluB-2/GUS/NOS, RP6/GUS/NOS, PG5a/GUS/NOS, Glb/GUS/NOS, Bx7/GUS/NOS, for the *Gt3*, *GluB-1*, *GluB-2*, *PG5a*, *RP6*, *Glb* and *Bx7* promoters, respectively.

Table 1. Primer sequence used to amplify promoter fragment

Primers	Primer sequences	PCR amplified fragment (bp)	Annealing temperature
Gt3/fw Gt3/rv	GTTAGTcTGCAgTGTAAGTGTAGCTTC ATGGTTGtCtaGaTTTTGTGGGACTGAAC	856	58°C
GluB-1/fw2 GluB-1/rv2	ACAGACAGcTGcAGAGATATGGATTTTCTAAG GGAACTCtCtAgAGCTATTTGTACTTGCTTATG	1319	62°C
GluB-2/fw GluB-2/rv	TCCGAGctgcAGTAATGGATACCTAGT GTAGTTtCtAgAGCTATTAGCAGTTGC	1O28	58°C
PG5a/fw2 PG5a/rv2	CGGTGcTGcAGATGGGTTGGGAACCCT ATGATCTagATTGCTCTGGGACATAGAT	874	58°C
RP6/fw RP6/rv	AATTCCTgCagCATCGGCTTAGGTGTA TGATCTagATTGTTGTTGGATTCTACT	684	58°C
Osglb/fw2 OSglb/rv	GGCGCCTGcAGGGAGGAGAGGGGAGAGAT ACCTTGCTctagATTGATGATCAATCAGA	997	58°C
Bx7/fw2 Bx7/rv	CGTCGTCTcTGcAGGCCAGGGAAAGACAATG CGCTTAtCtAgaTCAGTGAACTGTCAGTG	993	62°C

All transcription factor ("effector") plasmids where the coding sequences were placed under the control of the CaMV 35S promoter were generated by subcloning cDNA fragments of either O2, o2676 or PBF downstream of the CaMV 35S promoter and the *Adh1* intron and upstream of the *nos* 3' end in pMF6. For O2, the *Bgl*II O2Δl cassette described by Schmidt *et al.*, 1992 (which removes the start codons for the three small ORFs present in the 5' leader sequence, thus promoting increased O2 expression), was inserted into the *BamH*I site of pMF6. The o2676 effector was generated as described for O2 except that an internal restriction fragment containing the o2676 point mutation (Aukerman, 1991) was substituted for the corresponding restriction fragment in *Bgl*II O2Δl cassette. For PBF, a *BamH*I-*Xho*I fragment containing the entire PBF cDNA (VicenteCarbajosa, 1997) was subcloned into the same sites of pMF6. O2Δ1 and PBF were also expressed under the control of the maize *UBI1* promoter and first intron by subcloning the respective cDNA clones into the BamHI site of the pAHC17 plasmid (Christensen *et al.*, 1996).

Figures 11A-C are a schematic depiction of plasmids containing the (A) barley prolamin box binding factor protein (BPBF), (B) maize prolamin box binding factor protein (PBF) and (C) the maize opaque2 binding protein (O2) transcription factor coding sequences under the control of the rice endosperm-specific glutelin promoter (Gt-1).

The construction of antisense plasmids for the O2 and PBF DNA binding domains was carried out using PCR primers designed to amplify the highly conserved region of DNA binding domains of O2 and PBF. The PCR primer sets for *O2* were (M*O2*/fw: 5'-TTCTGGGATCCAAGATGCCTACCGAGG-3' (SEQ ID NO:17) and M*O2*/rv: 5'-GGGGTCGGATCCGAGATGGGCATGGAC-3' (SEQ ID NO:18), and for PBF (PBF/fw: 5'-AGTGGGGATCCTAAGCCGAGGCCGCAAC-3' (SEQ ID NO:19) and PBF/rv: 5'-GCTAGGGGATCCTGGTGCATAGGTAGCA-3' (SEQ ID NO:20), resulting in amplification of 333 bp and 278 bp amplification fragments, respectively when *Ubi*:*O2* and *Ubi*:PBF were used as the template.

The PCR reactions were performed in 50 µl of 1X PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, and 0.1 % Triton X-100) containing 250 µM dNTP, 1 ng of template, 0.1 µM of each of forward and reverse primers, and 0.3 unit of *Taq* polymerase (Perkin-Elmer). The GeneAmp PCR system (model 2400, Perkin-Elmer) was programmed for an initial denaturing temperature of 94 °C for 4 min, a 30 sec denaturing temperature of 94 °C, an annealing temperature of 58 °C for 30 sec, and an extension temperature of 72 °C for 2 min. The reaction was carried out for 30 cycles. An additional extension at 72 °C followed for 5 min was allowed to proceed after completion of the final cycle. The PCR products were purified by phenol:chloroform:isoamyl alcohol extraction procedure and precipitated with 100 % ethanol. After resuspending in 50 µl of dH20, the amplified products were digested with *BamH*I. The

BamHI flanked PCR products were used to replace the luciferase coding region of pAHC18 [Christensen *et al.*, 1996].

Insertion of the PCR-amplified DNA binding domain of *O2* and PBF in antisense orientation into the expression cassette containing the ubiquitin promoter was completed by PCR using the primer sets M*O2*/rv and NOS/rv; 5'-CGGCAACAGGATTCAATCT-3' (SEQ ID NO:21), PBF/rv and NOS/rv. The PCR was performed under the conditions described above, with the exception that the annealing temperature was changed to 53 °C.

B. Transient Assay using Rice Endosperm

DNA coated gold particles were prepared by mixing 50 µl of gold suspension (60 mg/ml), 50 µl of $CaCl_2$, 2.5 M and 20 µl of spermidine, 0.1 M. In all cases, 5 µg of the GUS chimeric construct and 5 µg of pAHC18 (containing the luciferase gene under the control of the ubiquitin promoter), were used. For co-transfection with effector plasmids, effector plasmids were additionally added in the amount indicated. The total amount of plasmids used for coating gold particles remained constant by adding the pBluescriptII KS (+) or the PMF plasmid (containing the *CaMV 35S* promoter driving an expression cassette which lacks a coding region). After vortexing for 1 min, the gold particles were washed with 100 % ethanol twice and finally resuspended in 50 µl of 100 % ethanol. 10 µl of gold particle suspension was loaded into a macrocarrier for bombardment.

Rice immature seeds at 7 to 9 DAP (days after pollination) were harvested and sterilized with husk by incubating 10 minutes in 70 % ethanol and followed by spraying with 100 % ethanol. After the ethanol evaporated completely, transient assay incubation buffer (TAIB: complete AA medium [Thompson, 1986] supplemented with NH_4NO_3 1.4 g/L, 100µg/ml of cefotaxime and 100 µg/ml of timentin) was added to prevent seeds from drying out. A portion of each seed grain which contains the embryo (about one fifth) was cut off using a sharp blade and the immature endosperm was squeezed out. About 10 rice immature endosperms were placed in the center of a 3 MM Whatman filter paper, prewetted with TAIB. Particle bombardment was carried out using the biolistic helium gun device (Dupont PSD-1000), as described Hwang *et al.*, 1998. After bombardment, the bombarded immature endosperms were incubated in 5 ml of TAIB in the dark at 25 °C for 1 day.

After incubation, immature endosperms were transferred to a conical Eppendorf tube and homogenized in 55 µl of extraction buffer (KH_2PO_4, 0.1 M, EDTA, 1 mM and β-mercaptoethanol, 7 mM) by a disposable plastic pestle. After spinning down the cell debris by centrifuging at 15,000 rpm for 15 min, 20 µl of supernatant was used in an assay for GUS or luciferase enzyme activity [deWet, 1987; Bronstein, 1994].

C. Transcriptional Activation with O2 and PBF

Transient expression assays were carried out to evaluate the effector activity of the O2 and PBF transcription factors on GUS expression.

Rice immature endosperms were isolated from caryopses at 7-9 DAP (DAP:Days After Pollination). The amount of each effector plasmid used is indicated in the individual examples, below. The total amount of plasmid for coating the gold particles remained constant by adding pBluescriptII KS(+). To normalize transfection efficiency, in every experiment, a luciferase gene under the control of the maize ubiquitin promoter was co-transfected (with 5 μg of pAHC18 (Ubi/LUC/NOS) used as an internal control for all experiments). Following measurements of GUS and LUX activity, the GUS expression level was normalized by dividing by the LUX activity from luciferase to obtain the GUS/LUX ratio. Therefore, the GUS/LUX ratio quantitatively indicates the transcriptional activity from the promoter of the heterologous reporter gene.

Developing rice endosperms at 7-9 DAP were biolistically bombarded with various heterologous nucleic acid constructs to examine the ability of the maize trans acting factor genes encoding opaque 2 (O2) and prolamin box binding factor (PBF) to effect expression of heterologous nucleic acid constructs under the control of the promoter regions from the rice glutelin genes Gt3, GluB-1 and GluB-2; the rice prolamin genes RP6 and PG5a; the rice globulin gene Glb; and the wheat glutelin gene, Bx7. O2 and PBF were expressed under the control of the Ubi promoter in the Ubi:O2 and Ubi:PBF constructs, respectively.

As shown in Figure 13B, co-transfection of the *Gt*1 reporter plasmid (Gt1/GUS/NOS) with *35S:O2* and *35S*:PBF resulted in approximately a 5 and 3-fold in transcription activity, respectively, relative to the activity from the *Gt1* reporter plasmid alone (Figure 13B).

When immature endosperms were co-bombarded with an equimolar mixture of the *35S:O2* and *35S*:PBF constructs, GUS expression from Gt1/GUS/NOS increased up to 6-fold (Fig. 14B). Transient assays using effector plasmids expressing mutant forms of the O2 and PBF transcription factors demonstrated that the observed activation is a consequence of direct interaction between O2 and PBF and the *Gt1* promoter. The relative GUS/LUX ratio from the *Gt1* reporter plasmid was not affected by co-bombardment with effector plasmids expressing defective forms of the O2 and PBF proteins (Fig. 14B) indicating that the capability of O2 and PBF to bind their specific target sites is critical for transactivation of the *Gt1* promoter.

Figure 13B illustrates transactivation of the *Gt1* promoter by various amounts of O2 and PBF effector plasmids in rice immature endosperms. As shown in Fig 13B, *Ubi:O2* and *Ubi*:PBF at the amount of 1 μg were able to transactivate the *Gt1* promoter, approximately 4 and 3 fold, respectively. This transactivation effect increased along with the increase in the amount

of effector up to approximately 5 μg. The increase in promoter activity by O2 and PBF together was shown to be additive, independent of the amount of plasmid combined (Fig. 13B).

As described above, in addition to the promoter of rice glutelin gene (*Gt1*), several other promoters of genes encoding different kinds of seed storage proteins like rice globulin, rice prolamin and wheat glutenin were tested for their responsiveness to O2 and PBF (Fig. 12A). The transcription activity from the promoter of rice globulin, *Glb* was increased by about 3 and 4 fold, in the presence of O2 and PBF, respectively (Fig. 12B). *Bx7*, the glutelin promoter from wheat, was shown to be transactivated in rice immature endosperms by co-bombardment with O2 and PBF up to about 1.8 fold, respectively (Fig. 12B). The O2 and PBF effectors were also shown to transactivate the rice prolamin gene promoters, *RP6* about 5 and 3.5 fold, and *PG5a*, about 2 and 1.5 fold, respectively (Fig. 12B).

The effect of O2 and PBF on the promoters from different kinds of rice storage genes including *Gt1*, *Gt3*, *GluB-1* and *GluB-2*; the rice prolamin genes *RP6* and *PG5a*; the rice globulin gene *Glb* and the wheat glutelin gene, *Bx7* was tested. Table 2 shows the responsiveness of the promoters to O2 and PBF, with an additive increase in transactivation observed by co-transfection with both effector plasmids. Of the promoters tested, the *Gt1* promoter was the most responsive to both of O2 and PBF and co-transfection with both plasmids gave about a 15 fold increase in GUS activity. The additive increase in promoter activity by co-bombardment of both effector plasmids was observed in all the promoters of storage protein genes that were tested. The rice actin promoter was much less responsiveness to O2 and PBF effectors and the promoter activity of the *CaMV 35S* promoter was not affected by co-transfection with O2 and PBF effector plasmids (Table 2).

Table 2. GUS expression in rice immature endosperms in response to O2 and PBF.

Effector	O2[1]	PBF[1]	O2/PBF[1]
Gt1	10.8(2.14)	4.57(1.55)	14.6(1.08)
Glb	3.22(0.72)	4.42(0.85)	8.55(2.27)
RP6	5.22(1.45)	3.36(0.71)	8.20(1.18)
Bx7	1.78(0.13)	1.84(0.38)	3.81(1.03)
PG5a	2.13(0.42)	1.59(0.18)	3.14(0.60)
Actin	1.59(0.25)	1.14(0.21)	1.64(0.48)
CaMV 35S	1.18(0.04)	1.06(0.18)	1.34(0.12)

[1] fold activation was calculated by normalizing the GUS/LUX ratio from rice endosperms co-bombarded with each effector and a reporter construct to the GUS/LUX ratio of that effector alone. For example, for GT1, all results are given relative to the GUS/LUX ratio of Gt1/GUS/NOS construct.
S.D. indicates the standard deviation of a mean value for at least five independent particle bombardments.

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S.D. indicates the standard deviation of a mean value for at least five independent particle bombardments.

[1] Results are presented as "fold activation(SD)", calculated by normalizing the GUS/LUX ratio from rice endosperms co-bombarded with each effector and a reporter construct to the GUS/LUX ratio of that effector alone. For example, for GT1, all results are given relative to the GUS/LUX ratio of Gt1/GUS/NOS construct.
S.D. indicates the standard deviation of a mean value for at least five independent particle bombardments.

EXAMPLE 4

Generation of Transgenic Plants Which Express Plant Transcription Factors

Heterologous nucleic acid constructs were prepared for generation of stable transgenic plant lines. Figs. 15A and B present a schematic depiction of exemplary plasmids for use in generating such stable transgenic plants. The plasmids contain a heterologous protein coding sequence for lactoferrin and lysozyme under the control of the rice endosperm-specific globulin promoter (Glb), as shown in Fig. 15A and Fig. 15B, respectively.

A. Vector Constructs

A gene encoding the mature polypeptide of human lysozyme (EC 3.2.1.17) with a G+C content of 68.4 % was synthesized (Operon, Alameda, CA, USA) based on the sequence of GenBank Accession number J03801. The DNA was digested with *DraI/XhoI*, and ligated into the *NaeI/XhoI* sites of the expression cassette in plasmid API241 which contains the rice globulin gene promoter and signal peptide (GenBank Accession Number X63990). The resulting plasmid was named pAPI264.

B. Development of Stable Transformants and Transgenic Plants

Microprojectile-mediated transformation of rice was carried out based on the procedure described in [Chen, 1998]. Calli were derived from the hypocotyls of germinating mature seeds of the cultivar TP309 (*Oryza sativa*, subspec. *Japonica*). Calli with a diameter of 2-4 mm were selected and placed on a N6 medium (Sigma, Louis, MO, USA) supplemented with 0.3M mannitol and 0.3M sorbitol for about 20 hours before bombardment. Bombardment was carried out with the biolistic PDC-1000/He instrument (Bio-Rad, Hercules, CA, USA). Fifty µl of gold particles (60mg.ml^{-1} at 1:1 ratio of 1.0 and 1.5-3.0 µm diameter gold particle) were coated with effector DNA, target DNA and selection marker DNA in a ratio of 3:3:1 (w/w) and accelerated with a helium pressure of 1100 psi. After two day's incubation, calli were transferred to N6 selection media containing 35mg·l^{-1} hygromycin B and allowed to grow in the dark at 26°C for 45 days. Calli resistant to hygromycin B were transferred to regeneration media and used to generate plantlets as described in [Chen, 1998]. After shoots had reached a height of 1-3 cm, the plantlets were transferred to rooting media (MS plus 0.05 mg·l^{-1} α–Naphthaleneactic acid, Sigma, Louis, MO, USA) and two weeks later the plantlets were transferred to soil and grown in the greenhouse to maturity.

C. PCR Analysis of Transgenic Plants

Genomic DNA was prepared from samples of transgenic plant leaves as described in [Dellaporta, 1983 59] and used as the template for amplification with two pairs of primers for the

identification of transgenes. For the rice Reb gene, the forward primer 5'-CCATCCAATCCAATCCACTCCAAC-3' (Fig. 16A; SEQ ID NO:22) was designed based on a 3' untranslated terminal sequence of the gene, and the reverse primer was designed based on the vector sequence 5'-AGGCGATTAAGTTGGGTAACG-3' (Fig. 16A, SEQ ID NO:23). For the human lysozyme transgene, the forward primer was designed based on the 5' end of the open reading frame of the gene 5'-CCTAGCCAAAGTCTT CGAGCGGTG-3' (Fig. 16B; SEQ ID NO:24), and the reverse primer was designed based on the 3' end of the open reading frame of the gene 5'-GCGATGTTGTCTTGCAGC-3' (Fig. 16B; SEQ ID NO:25). The PCR mixture contained 100 ng genomic DNA, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.5 μM dNTP. Amplification employed a program of denaturing at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds. The PCR products were resolved by electrophoresis in a 1.2 % agarose gel.

D. Lysozyme Activity Assay

A lysozyme assay was carried out using a procedure where twenty individual seeds from each T1 transgenic plant were ground in 1 ml of pre-cold extraction buffer (PBS plus 0.35 M NaCl). After centrifugation at 25,000g for 5 min at 4°C, the supernatant was recovered. A series of dilutions were made and an aliquot was added to a 96-well microtiter plate containing 250 μl of 0.015 % *Micrococcus letus* cells in each well (Sigma, Louis, MO, USA; procedure developed at Applied Phytologics Inc.). Human lysozyme (*EC 3.2.1.17*, Sigma, Louis, MO, USA) was used as the standard and lysozyme activity was measured based on the decrease in turbidity, evaluated using a Microplate Reader 3550 (Bio-Rad, Hercules, CA, USA). The lysozyme concentration in the samples was determined based on absorbance values of samples relative to a standard curve prepared using different concentrations of human lysozyme. The lysozyme expression level in a given transgenic plant was calculated as the average lysozyme content of the twenty seeds taken from that plant. Total soluble protein in seed extracts was estimated using the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

E. Enhanced Human Lysozyme Expression in Transgenic Rice Seed Co-Transformed with Reb

In order to evaluate the ability of Reb to increase the expression of a transgene in transgenic plants, plants cells were co-transformed with heterologous nucleic acid constructs comprising the human lysozyme gene driven by the Glb promoter (Glb-lys) and the Reb gene driven by the actin promoter (Act-Reb) and with the Act-Reb construct alone. Normal plant phenotypes were obtained among transformants containing both Glb-lys and native Reb and PCR

analysis confirmed that both genes had integrated into the genome in 10 out of 11 plants (Fig. 17). Transgenic seeds were harvested at maturity and lysozyme activity was analyzed.

Lysozyme expression in seeds of the 10 transgenic plants containing native-Reb and Glb-lys ranged from 31-133 $\mu g \cdot mg^{-1}$ soluble protein with an average of 69.8±11.6 $\mu g \cdot mg^{-1}$ soluble protein (Fig. 17). Seeds taken from seventeen transgenic plants containing Glb-Lys alone expressed lysozyme in amounts ranging from 7 to 76 $\mu g \cdot mg^{-1}$ soluble protein with an average of 33.95±4.96 $\mu g \cdot mg^{-1}$ soluble protein (Fig. 17). No lysozyme activity was detected in untransformed rice seeds (Fig. 17). The results showed that the expression level of the lysozyme increased on an average 2-fold, when the seeds were transgenic for both the Reb gene and Glb-lys. The statistical analysis (t-test) showed that the amount of lysozyme in seeds from the plants transgenic for the Reb gene and Glb-lys is significantly higher than in the plants with Glb-lys alone ($P<0.001$).

Table 3. Brief Description of the Sequences.

Description	SEQ ID NO
forward PCR primer for REB BAC DNA: 5'-CTGATATGTGCCCATGTTCCAAAC-3'	1
reverse PCR primer for REB BAC DNA: 5'-CCTTGCTGAATGCAGATGTTTCAC -3'	2
Gt3/fw primer:GTTAGTcTGCAgTGTAAGTGTAGCTTC	3
Gt3/rv primer:ATGGTTGtCtaGaTTTTGTGGGACTGAAC	4
GluB-1/fw2 primer:ACAGACAGcTGcAGAGATATGGATTTTCTAAG	5
GluB-1/rv2 primer:GGAACTCtCtAgAGCTATTTGTACTTGCTTATG	6
GluB-2/fw primer:TCCGAGctgcAGTAATGGATACCTAGT	7
GluB-2/rv primer:GTAGTTtCtAgAGCTATTAGCAGTTGC	8
PG5a/fw2 primer:CGGTGcTGcAGATGGGTTGGGAACCCT	9
PG5a/rv2 primer:ATGATCTagATTGCTCTGGGACATAGAT	10
RP6/fw primer:AATTCCTgCagCATCGGCTTAGGTGTA	11
RP6/rv primer:TGATCTagATTGTTGTTGGATTCTACT	12
Osglb/fw2 primer:GGCGCCTGcAGGGAGGAGAGGGGAGAGAT	13
OSglb/rv primer:ACCTTGCTctagATTGATGATCAATCAGA	14
Bx7/fw2 primer:CGTCGTCTcTGcAGGCCAGGGAAAGACAATG	15
Bx7/rv2 primer:CGCTTAtCtAgaTCAGTGAACTGTCAGTG	16
forward PCR primer for O2 DNA M*O2*/fw: 5'-TTCTGGGATCCAAGATGCCTACCGAGG-3'	17
reverse PCR primer for O2 DNA M*O2*/rv: 5'-GGGGTCGGATCCGAGATGGGCATGGAC-3'	18
forward PCR primer for PBF DNA PBF (PBF/fw: 5'-AGTGGGGATCCTAAGCCGAGGCCGCAAC-3'	19
reverse PCR primer for PBF DNA PBF/rv: 5'-GCTAGGGGATCCTGGTGCATAGGTAGCA-3'	20
NOS/rv PCR primer CGGCAACAGGATTCAATCT	21
forward primer for REB analysis (Fig 18A) 5'-CCATCCAATCCAATCCACTCCAAC-3'	22
reverse primer for REB analysis (Fig 18A) 5'-AGGCGATTAAGTTGGGTAACG-3'	23
forward primer for human lysozyme analysis (Fig 18B) 5'-CCTAGCCAAAGTCTT CGAGCGGTG-3'	24
reverse primer for human lysozyme analysis (Fig 18B) 5'-GCGATGTTGTCTTGCAGC-3'	25
Oryza sativa glutelin 1 (Gt1) upstream regulatory sequence described in: Okita TW *et al.*, J. Biol Chem. 264:12573-12581, 1989 and GenBank Accession No. M28156	26
Oryza sativa prolamin *RP6* upstream regulatory sequence described in: Wen TN, *et al.*, Plant Physiol 101:1115-1116, 1993 and GenBank Accession No. X65064	27
Oryza sativa prolamin *PG5a* upstream regulatory sequence described in: Nakase M *et al.*, Plant Mol. Biol. 32:621-630, 1996 and GenBank Accession No. D73383)	28
Oryza sativa globulin gene *Glb* promoter sequence (Fig. 9)	29
Tricticum aestivum Bx7 gene promoter sequence (Fig. 10)	30
the Opaque2 coding sequence found at GenBank Accession No. X15544 (gene)	31
the Opaque2 coding sequence found at GenBank Accession No. M29411 (opaque2 cDNA).	32
rice PBF coding sequence found at GenBank Accession No. D11385	33
maize PBF coding sequence found at GenBank Accession No. ZMU82230	34
DNA coding sequence for the rice (*Oryza sativa*) Reb bZIP protein (Figures 2A-I)	35